# Complete acid based hydrolysis assay for carbohydrate quantification in seaweed: A species specific optimised approach

**Emily T. Kostas, Stuart J. Wilkinson, Daniel A. White and David J. Cook**

**Abstract**

Accurate quantification of the carbohydrate content of biomass is crucial for many bio-refining processes. The most commonly followed protocol is typically a modification of the NREL based assay (specifically designed for carbohydrate analysis from lignocellulosic biomass). However this NREL protocol was revealed to be excessively thermo-chemically harsh for seaweed biomass. This can result in erroneously low total sugar quantification as the reaction severity can degrade a proportion of the liberated sugars to decomposition products such as furans. Here we describe an optimisation of the total acid hydrolysis protocol for accurate quantification of the carbohydrate content of seaweeds. Different species of seaweed can be accurately evaluated for their carbohydrate contents by following this optimised method.

**Key Words:**

Macroalgae, Carbohydrate, Optimisation, Species, Composition, Mass Balance

Running head:

**carbohydrate quantification in seaweed**

## 1 Introduction

In order to measure the carbohydrate content of seaweed biomass, modified versions of the NREL two-stage acid hydrolysis protocol [1] are typically applied [2-4]. The NREL carbohydrate assay is composed of 2 distinct stages and was initially developed for the quantification of total carbohydrate in lignocellulosic biomass (Figure 1). The first stage of the assay is a low temperature (37°C) treatment with concentrated acid (typically 12 M $H_2SO_4$) which induces the initial swelling of the biomass followed by the fragmentation of the larger polysaccharide complexes found in the biomass. The second stage of the protocol involves using dilute acid (1 M $H_2SO_4$) but at a higher temperature (100°C) which then fully hydrolyses the larger sugar fragments (oligosaccharides) into their constituent monomeric units (monosaccharides). These monosaccharide sugars can then be quantified either by high performance liquid chromatography or gas chromatography mass spectrometry (HPLC or GC-MS, respectively) or alternatively via colorimetric methods [5]. Seaweed polysaccharides are distinctly different to those of terrestrial plants in terms both of the sugar subunits (monomers) which are present and also the specific linkages between the monomers. In addition seaweed-derived biomass is significantly less recalcitrant in nature when compared to lignocellulosic biomass. As such the NREL based protocol (which is specifically designed for lignocellulosic biomass) may be too thermo-chemically extreme for seaweed biomass. Therefore use of the NREL assay in its original format may significantly underestimate the 'true' carbohydrate content of the seaweed [6] through the potential degradation of liberated sugars into furan-based compounds [7]. We therefore evaluated the NREL protocol [1] to assess its suitability towards seaweed biomass and confirmed it to be thermo-chemically harsh [6]. Furthermore, we revealed that different species of seaweed require specific

individual optimisations of the protocol for accurate total carbohydrate quantification. From our experimental work using *Laminaria digitata* (which was used as a benchmark species), we identified that optimisation of stage 1 of the protocol (the low temperature and concentrated acid phase) had a greater impact on the assay than was evident for stage 2 (the high temperature dilute acid phase). The optimal conditions for obtaining the maximal sugar yields from *Laminaria digitata* required the use of 11 M $H_2SO_4$ originally rather than the 12 M $H_2SO_4$ used in the NREL protocol (Figure 2) as this reduced the degree of furan generation.

However, our experimental work concluded that stage 2 of the original NREL assay was already optimal and as such was not modified in any way (Figure 3). The newly optimized stage 1 conditions were then combined with the original NREL stage 2 conditions to formulate an optimised carbohydrate assay (for *L. digitata*). The subsequent application of this newly optimised (specifically for *L. digitata*) carbohydrate assay to further seaweed species (*Chondrus crispus* and *Ulva lactuca*) also produced higher total sugar yields and lower levels of sugar degradation products than when using the original NREL assay on the same biomass (Figure 4) even without any additional optimisation for each species. This suggested that further comprehensive optimisation of the assay for each individual species might liberate even higher total sugar yields. Overall this demonstrated the likely importance of specific individual optimisations of the protocol for each different species of seaweed for accurate total sugar quantification. Here we demonstrate a simple yet effective experimental methodology to help determine the optimum parameters (for stage 1 of the acid hydrolysis protocol) for the accurate quantification of carbohydrate content in any species of seaweed.

## 2 Materials

Prepare all reagents, solutions and perform all dilutions using ultrapure reverse osmosis (RO) water to achieve a sensitivity of >18MΩ-cm (at 25°C) and using analytical grade reagents unless otherwise stated. Caution must be used when handling hazardous reagents such as concentrated acid and phenol (if using the colourimetric sugar quantification). In addition we advocate the use of a fume hood or cabinet when dispensing such hazardous reagents.

### 2.1 Total acid hydrolysis and sugar-degradation products

1. Seaweed biomass (see Note 1)
2. Fan assisted oven
3. Ball mill or grinder (see Note 2)
4. 50 mL screw-capped Pyrex reaction tubes
5. Analytical balance
6. $H_2SO_4$, (72% v/v) (see Notes 3 and 4)
7. Water bath (or incubator), keeping 37°C and 100°C
8. Luer-lok type syringe
9. Syringe filter, luer-lok, <45 µm retention size
10. HPLC vials
11. 15 mL plastic centrifuge tube
12. HPLC system with UV detection at 270-290 nm
13. HPLC column; C18 Techsphere ODS column (5 µm, 4.6 mm × 250 mm; HPLC Technologies, UK) at ambient temperature and using gradient elution
14. Acetic acid (solvent)

15. Methanol (solvent)
16. Furoic acid, 0.1–1.0 g/L (standard)
17. Furfural, 0.1–1.0 g/L (standard)

2.2 **Total reducing sugars using a colorimetric assay**

1. HPAEC system with pulsed amperometric electrochemical detection (PAD)
2. HPAEC column, pa20 column (150 mm × 3.0 mm; Dionex, USA)
3. NaOH, 10 and 200 mmol / l
4. Mannitol solution, 0.0625 g/L - 1 g/L (standard)
5. Fucose solution, 0.0625 g/L - 1 g/L (standard)
6. Galactose solution, 0.0625 g/L - 1 g/L (standard)
7. Arabinose solution, 0.0625 g/L - 1 g/L (standard)
8. Galactose solution, 0.0625 g/L - 1 g/L (standard)
9. Glucose solution, 0.0625 g/L - 1 g/L (standard)
10. Xylose solution, 0.0625 g/L - 1 g/L (standard)

**2.3. Quantification of sugars by HPAEC-PAD**

1. Phenol solution, 2.5% (w/v) (see Note 5)
2. $H_2SO_4$, (72% v/v) (see Notes 3 and 4 )
3. Quartz cuvettes
4. Spectrophotometer
5. Glucose solution, 0, 200, 400, 600, 800 and 1000 µg/mL (standard)

## 3 Methods

Carry out all procedures at room temperature unless otherwise specified.

### 3.1 Total acid hydrolysis assay

1. Accurately weigh 30 mg of biomass into a 50 mL screw-capped Pyrex reaction tube (Pyrex, UK) using an analytical balance accurate to 4 decimal places.
2. Carefully add 1 mL of the required concentration of $H_2SO_4$ (suggested acid concentration range is 3 M -12 M; see section 2.2 for guidelines) into each reaction vessel (see note 6) ensuring the biomass is completely covered or submerged within the acid. Carefully ensure the lids are tight on all reaction vessels whilst ensuring that the biomass stays submerged within the acid.
3. For stage 1 of the assay place all reaction vessels in a test tube rack and place the rack in a 37°C water bath (or incubator) for 1 h (see note 7).
4. Upon completion of the 1 h incubation period at 37°C, remove the test tube rack and add the required aliquot of RO water (see note 8) to achieve dilution of the acid concentration to 1 M.
5. For stage 2 of the assay, carefully return the reaction vessel caps, return the vessels to the test tube rack and then place the rack in the 100°C water bath (or incubator) for 2 h.
6. After 2 h incubation at 100°C remove the test tube rack and place it into an ice-cold water bath (or similarly suitable tray) to cool and allow any particulates or suspended biomass to settle.
7. After cooling unscrew the reaction vessel caps and carefully remove an aliquot (ca. 2 mL) of the liquid phase using a disposable plastic Luer-lok type syringe (Becton-Dickinson, USA; 2-5 mL capacity typically) whilst attempting to minimise uptake of solid particles.

8. Syringe filter each sample into a clean glass test tube, using a Luer-lok type syringe filter (Whatman, UK) of <0.45 µm retention size.
9. For the HPLC quantification of sugar degradation products (see section 3.2 for details) remove an aliquot (0.5 mL – 1.0 mL) of each of the syringe-filtered samples and place them into the appropriate HPLC vials for the system to be used.
10. For the HPAEC-PAD quantification of sugars (see section 3.3) the syringe filtered samples will need to be diluted due to the sensitivity of the detection system as care must be taken not to overload the detector. The precise dilution factor required is dependent upon the concentration of the sugars within the sample, which is of course unknown at this stage. However, typically x1000 dilution is first evaluated (see Note 8).
11. For achieving a x1000 dilution for HPAEC-PAD: mix 100 µL of each of the filtered samples with 9.9 mL of RO water in a 15 mL plastic centrifuge tube (Fisher Scientific, UK) or a similar screw –capped test tube that can be inverted to ensure suitable mixing.
12. Transfer a 1 mL aliquot of this dilution into to a suitable HPLC vial for HPAEC-PAD analysis.
13. If using the more simple colorimetric-based determination of total reducing sugars; a 100 µL aliquot of each filtered sample is required (see section 3.4).

### 3.2 Quantification of sugar-degradation products by HPLC

1. This protocol utilises the method described in [8]. The HPLC system requires UV detection at 270-290 nm.
2. The use of a PDA (photo-diode array) variant of UV detection is highly recommended in order to provide additional spectral data to further aid the identification of any

peaks detected rather than relying purely on comparison of retention times with those of authentic standards.

3. The mobile phase is a binary mixture of 1% acetic acid (solvent A) and methanol (solvent B) running at a flow rate of 0.5 mL/min.
4. The gradient elution ramp is from 20% to 50% methanol over 30 min with a 100% methanol column cleaning phase (for 1 min) and a 9 min re-equilibration period (at 20% methanol) prior to the next injection.
5. The sample injection volume is 10 µL.
6. Quantification is performed by comparison of peak areas of authentic standards (0.1–1.0 g/L concentration range, dissolved directly in RO water) including 5-HMF, furoic acid and furfural (see Note 9).

### 3.3 Quantification of sugars by HPAEC-PAD

1. This protocol utilises the method described in [9].
2. Dilute samples x1000 prior to analysis (see section 3.1.11).
3. The HPAEC system uses pulsed amperometric electrochemical detection (PAD).
4. The system is operated using isocratic elution with 10 mM NaOH at 0.5 mL/min flow rate with a column regeneration step using 200 mM NaOH at 0.5 mL/min after each injection.
5. Quantification is performed by comparison of peak areas of authentic standards of mannitol, fucose, galactose, arabinose, galactose, glucose and xylose (0.0625 g/L - 1 g/L concentration range, dissolved directly in RO water; see note 10). Dilute standards x1000 (with RO water) prior to analysis.

### 3.4 Quantification of total reducing sugars using a colorimetric assay

1. This protocol utilises the phenol-sulfuric acid method described in [5].

2. Transfer a 100 µL aliquot of each sample into a 5 mL test tube.

3. Carefully add1 mL phenol solution (2.5% v/v) and 2.5 mL concentrated (72% v/v) $H_2SO_4$ (see note 11).

4. Transfer 2 mL aliquots of this reaction mixture into quartz cuvettes.

5. Read absorbances at 490 nm using a spectrophotometer (after zeroing the instrument using water as a blank).

6. Quantification is then achieved by comparison to the absorbance (at 490 nm) of authentic glucose standards (0, 200, 400, 600, 800 and 1000 µg/mL).

## 4 Notes

1. All seaweed biomass must be dried in a fan-assisted oven at ca. 80°C for a minimum of 48 h prior to use

2. Dried biomass must be ground up or milled to ensure adequate homogeniety prior to analysis. Ball-milling (or the use of a similar technique) is advised to produce a fine powder of the biomass that aids the acccurate weighing of small quantities of material. Once dried and milled, the biomass can be stored at room temperature in an air-tight container.

3. The suggested range of acid concentrations evaluated for optimisation of stage 1 of the total acid hydrolysis is 3 M – 12 M, with dilutions performed using RO water to prepare the reagents prior to use. For the optimisation of stage 1 of the protocol for your specific biomass type, we would recommend a minmum series of acid concentrations of: 3 M, 6 M, 9 M and 12 M (see note 1). However, a more comprehensive optimisation can be achieved using the experimental run conditions

219 outlined in Table 1. Store reagents in a hazardous chemical cabinet such as that
220 designed for flammables.

221 4. Concentrated $H_2SO_4$ is highly hazardous and we advocate all handling is conducted in
222 a fume hood (cabinet). In addition, the dilution process for the acid to achieve the
223 desired acid molarities is highly exothermic and care should be taken to allow
224 solutions to cool before use.

225 5. A 2.5% (w/v) phenol solution is prepared by adding 2.5 g phenol to 100 mL RO water
226 (see note 2). Phenol is also a highly hazardous compound and should always be
227 handled in a fume hood (cabinet) and store in a hazardous chemical cabinet once
228 prepared.

229 6. The use of automatic dispensette® pipettes (BrandTech Scientific, USA) is highly
230 recommended for the rapid, reproducible, and safe dispensing of reagents.

231 7. Set all water baths or incubators to the correct temperature prior to commencing the
232 assay as attemperation for the 100°C water bath may take >2 h.

233 8. Example dilution of acid from 12 M to 1 M would involve careful addition of 11 mL
234 RO water.

235 9. If using the x1000 dilution factor for samples (for HPAE-PAD based total sugar
236 analysis) and the subsequent dection response if poor (poor signal to noise ratio
237 through inadequately small peak sizes) then a more concentrated sample dilution can
238 be evaluated such as x100.

239 10. A large stock of HPLC and HPAEC-PAD standards may be produced, filtered
240 through < 0.45 μm filters, labelled and stored at -20°C for up to 3 months if frequent

241 analysis is required. The stock can be removed from the freezer, thawed and vortexed
242 prior to use.

243 11. The reaction of $H_2SO_4$ with phenol is highly exothermic therfore care should be taken.
244 We advocate all that all additions of regaent (reactions) are conducted in a fume hood.

## 5 References

1. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. NREL Laboratory analytical procedure 1617

2. van der Wal H, Sperber BL, Houweling-Tan B, Bakker RR, Brandenburg W, López-Contreras AM (2013) Production of acetone, butanol, and ethanol from biomass of the green seaweed Ulva lactuca. Bioresource technology 128:431-437

3. Ge L, Wang P, Mou H (2011) Study on saccharification techniques of seaweed wastes for the transformation of ethanol. Renewable energy 36 (1):84-89

4. Trivedi N, Gupta V, Reddy C, Jha B (2013) Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga Ulva fasciata Delile. Bioresource technology 150:106-112

5. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem 28 (3):350-356. doi:Doi 10.1021/Ac60111a017

6. Kostas ET, Wilkinson SJ, White DA, Cook DJ (2016) Optimization of a total acid hydrolysis based protocol for the quantification of carbohydrate in macroalgae. Journal of Algal Biomass Utilisation 7 (1):21-36

7. Wilkinson S, Smart K, Cook D (2014a) A comparison of dilute acid and alkali catalysed hydrothermal pre-treatments for bioethanol production from Brewers Spent Grains. J Am Soc Brew Chem 72 (2):143-153

8. Wilkinson S, Smart, K., Cook, D (2014b) Optimisation of alkaline reagent based chemical pre-treatment of Brewers Spent Grains for bioethanol production. Ind Crops Prod 62:219-227

9. Kostas ET, White DA, Du C, Cook DJ (2016) Selection of yeast strains for bioethanol production from UK seaweeds. Journal of applied phycology 28 (2):1427-1441

10. Wilkinson S, Smart KA, Cook DJ (2015) Optimising the (microwave) hydrothermal pretreatment of brewers spent grains for bioethanol production. Journal of Fuels 2015

**Figure 1 Overview of the NREL assay (Sluiter et al, 2008) for determining carbohydrate analysis of lignocellulosic biomass.**

Stage 1: 1 mL of 12M $H_2SO_4$ is added to biomass (30 mg) and incubated at 37 °C for 1h, liberating the larger polysaccharides from the biomass. Stage 2: acid strength diluted with distilled water to 1 M and incubated at 100 °C for 2 h which hydrolyses the polysaccharides into their monomeric constituents. Quantification then achieved either using chromatographic or colourimetric methods.

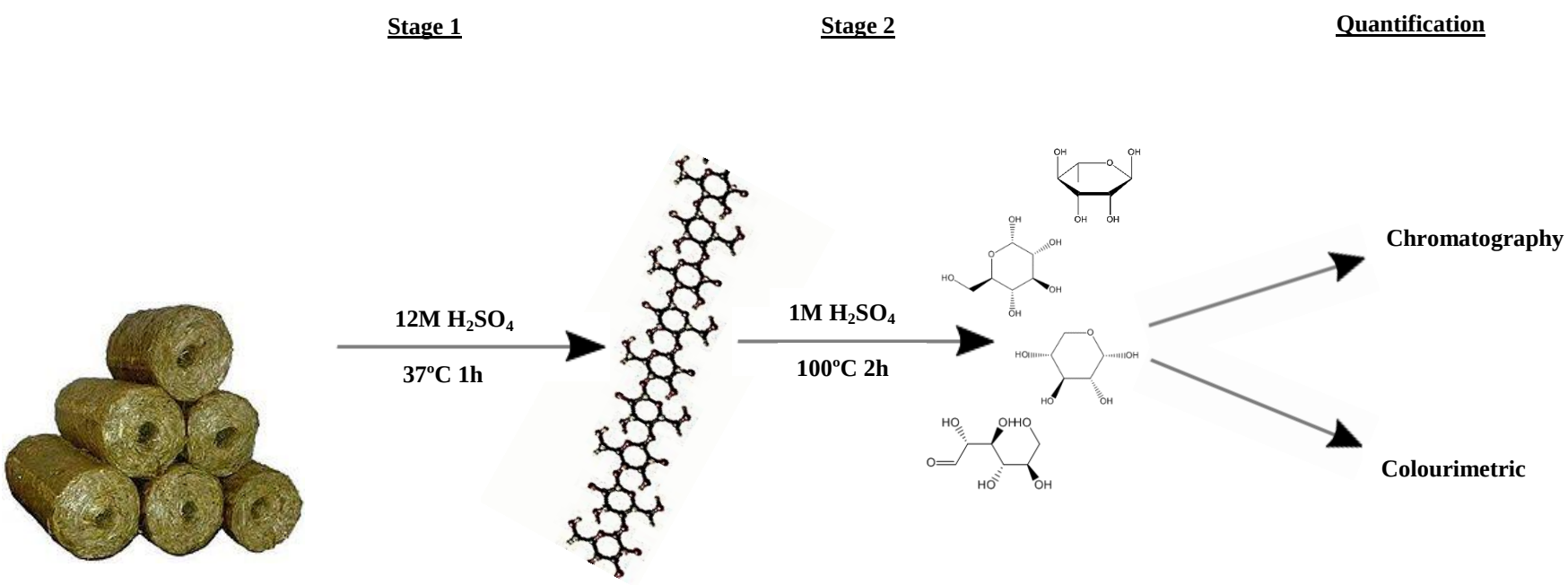

**Figure 2 3D response surface model showing the impact of different sulphuric acid concentrations (M) and reaction times (min) on the release of reducing sugars (mg/g) from *L. digitata*.**

Quantification using the phenol-sulphuric acid (Dubois) colourimetric assay. Model $R^2$: 0.56.

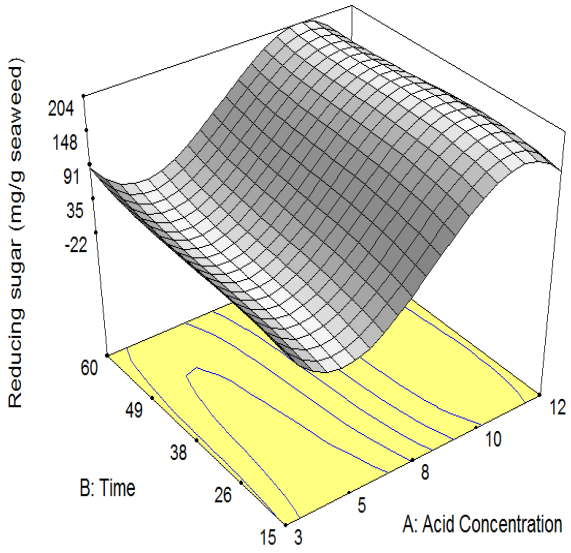

**Figure 3 3D response surface model showing the effect of simultaneous variation of acid concentration (M) and reaction temperature (°C) on the release of reducing sugars (%) from *L. digitata* during stage 2 of the total acid hydrolysis protocol.**

Stage 2 incubation time: 2 h. Reducing sugars quantified by phenol-sulphuric colourimetric assay. SCP involved using previously optimised stage 1 parameters: 11 M $H_2SO_4$, 37°C, 1 h. Model $R^2$: 0.17.

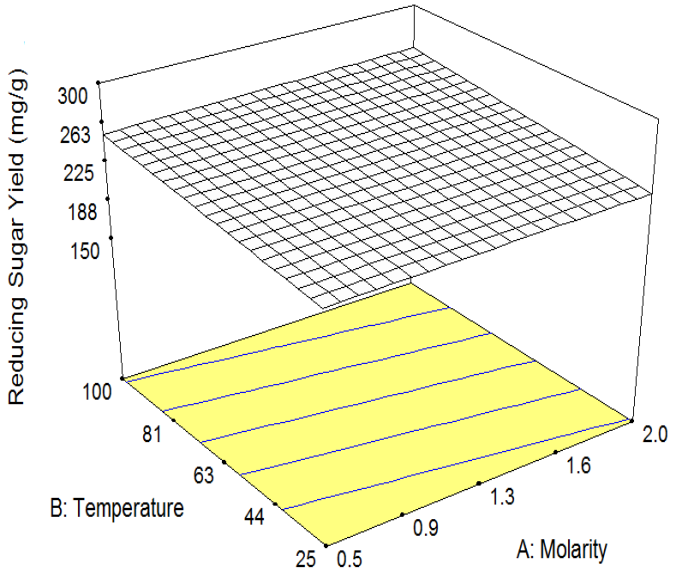

**Figure 4 Comparison between NREL assay and optimised carbohydrate quantification assay for total sugars measurement of the seaweed species *U. lactuca* and *C. crispus*.**

**A** Total sugar yields (sum of mannitol, fucose, arabinose, galactose, glucose and xylose; quantified by HPAEC-PAD) from both *U. lactuca* and *C. crispus*. **B** Furfural concentrations generated from both *U. lactuca* and *C. crispus* from both the original control protocol and the newly optimised protocol.

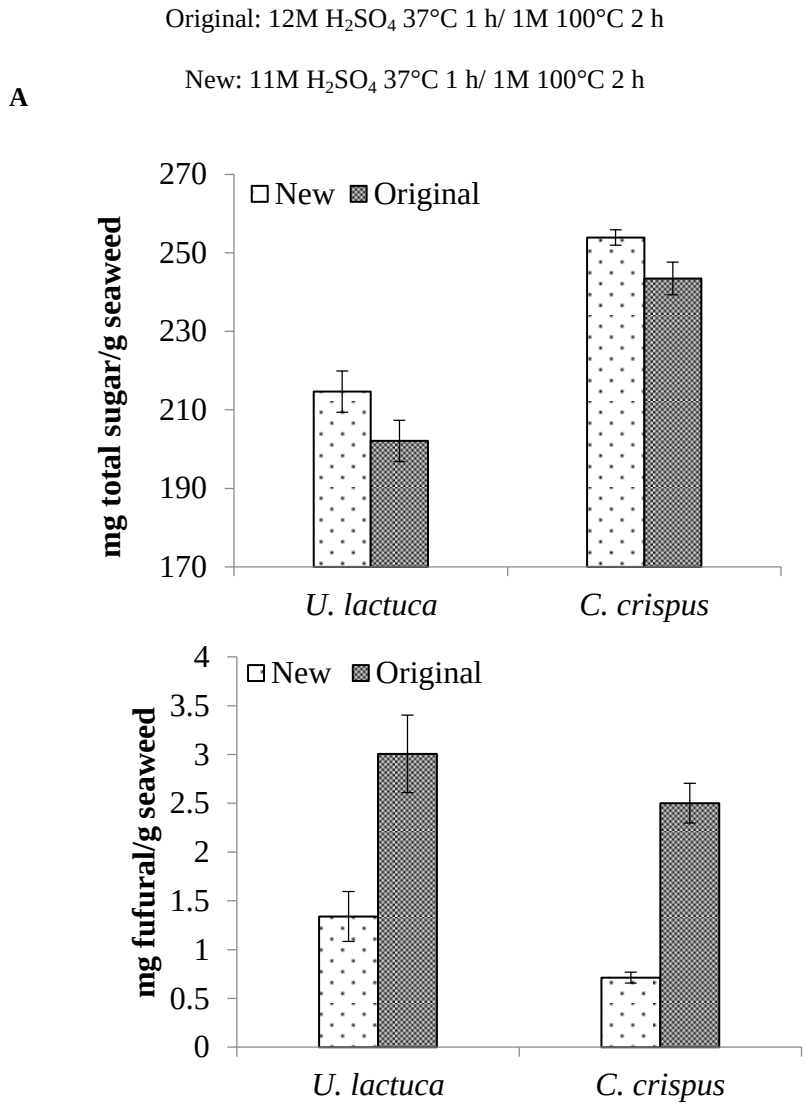

**Table 1 Experimental design used to optimise stage 1 (37°C) of the total acid hydrolysis methodology for quantifying carbohydrates in seaweed.**

Optimisation conducted through screening different sulphuric acid concentrations (3-12M) and reaction times (15-60 min) at 37°C, according to a *D-optimal* design space.

| Run | **Factor 1** A: $H_2SO_4$ Acid Conc (M) | **Factor 2** B: Time at 37°C (min) | Run | **Factor 1** A: $H_2SO_4$ Acid Conc (M) | **Factor 2** B: Time at 37°C (min) |
|---|---|---|---|---|---|
| 1 | 12 | 15 | 22 | 10 | 60 |
| 2 | 3 | 15 | 23 | 10 | 15 |
| 3 | 3 | 15 | 24 | 12 | 60 |
| 4 | 7 | 15 | 25 | 12 | 60 |
| 5 | 7 | 35 | 26 | 12 | 15 |
| 6 | 3 | 60 | 27 | 12 | 15 |
| 7 | 5 | 45 | 28 | 8 | 15 |
| 8 | 12 | 35 | 29 | 8 | 15 |
| 9 | 7 | 35 | 30 | 8 | 60 |
| 10 | 5 | 25 | 31 | 12 | 35 |
| 11 | 12 | 60 | 32 | 8 | 35 |
| 12 | 10 | 25 | 33 | 10 | 15 |
| 13 | 3 | 35 | 34 | 10 | 25 |
| 14 | 3 | 60 | 35 | 10 | 25 |
| 15 | 12 | 15 | 36 | 10 | 45 |
| 16 | 7 | 60 | 37 | 10 | 45 |
| 17 | 12 | 60 | 38 | 10 | 60 |
| 18 | 7 | 35 | 39 | 9 | 20 |

| | | | | | |
|---|---|---|---|---|---|
| 19 | 7 | 35 | 40 | 9 | 50 |
| 20 | 10 | 45 | 41 | 11 | 20 |
| 21 | 5 | 60 | 42 | 11 | 50 |